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Use of Fish Ventilation Frequency to Estimate Chronically Safe Toxicant Concentrations[1]

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Abstract

A 96-hour toxicity test with fish ventilation frequency as the response variable was developed to estimate safe zinc concentrations for steelhead *Salmo gairdneri*. Two Oregon steelhead strains were exposed to zinc at different water temperatures and total hardnesses (7, 12, and 17 C; 25 and 125 mg/liter as $CaCO_3$). Ventilation frequencies enumerated from bioelectric potentials generated by buccal and opercular openings and closings showed significant increases at the highest test concentration in five of 10 tests. At 12 C and 25 mg/liter hardness, responses were detected at a zinc concentration of 144 μg/liter; the "safe" concentration determined in a chronic exposure of embryos and juveniles was between 444 and 819 μg zinc/liter. This indicates that ventilation-frequency tests are at least as sensitive as long-term toxicity tests and may be used to screen chemicals for potential harmful effects on fish.

This study was designed to determine the effect–no-effect concentrations of zinc for steelhead *Salmo gairdneri* based on ventilation-rate monitoring. We developed a system to expose steelheads to zinc solutions in flow-through chambers so that ventilatory patterns could be measured with external electrodes. The system was evaluated for two steelhead strains under varying regimes of temperature and hardness. Minimum zinc concentrations that produced a change in fish ventilation rate during 96 hours were compared with the maximum acceptable toxicant concentration determined from chronic exposures of embryos and early juveniles (MATC[EL]) to judge the suitability of ventilation-response concentration (VRC) as a monitoring tool. Such a tool has three principal applications in water-pollution biology: screening of chemicals prior to marketing; biomonitoring of effluents; and development of criteria for water quality standards. Ventilation-rate monitoring has promise in these regards.

Estimates of chemical toxicity in the aquatic environment usually are based upon responses (growth, survival, et cetera) during acute and chronic toxicity tests. Chronic tests that embrace a full life cycle are the most common, most sensitive methods for estimating long-term no-effect concentrations (Stephan and Mount 1973). Results from chronic bioassays are used to compute an MATC (Mount and Stephan 1967), which is between the lowest concentration causing a significant effect and the highest concentration that does not cause an effect. However, the need for a scanning tool capable of rapid, reliable prediction is urgent because under the most favorable conditions chronic tests take at least 1 year (with salmonids). Evidence exists showing that embryo–early juvenile exposures produce reliable estimates of MATCs (Macek and Sleight 1977; McKim 1977).

Methods

The MATC(EL), as well as published median lethal (LC50) acute values and MATCs were used to evaluate the ventilation tests as predictors of safe chronic concentrations of zinc. Ten 96-hour ventilation-rate tests were run at three temperatures, at two hardnesses, and with two

[1] Technical Paper 5573, Oregon Agricultural Experiment Station; this paper contains material from a thesis submitted by M. A. C. to Oregon State University in partial fulfillment of the requirements of the degree of Master of Science.

strains of steelhead to determine the effects of these variables on test results.

Fertilized steelhead eggs were obtained from Oregon Department of Fish and Wildlife hatcheries on the day of spawning. The 1977 group was from the Alsea River; the 1978 group was from the North Santiam River. All eggs were dipped in Wescodyne[2] disinfectant solutions before incubation. At swimup, fish were transferred to troughs and later to larger tanks where they were fed Oregon Moist food; fish were not fed for 48 hours prior to or during ventilation tests.

Alsea River smolts used in ventilation tests had a mean (±SD) wet weight of 122.4 ± 21.2 g and a mean (±SD) fork length of 24.0 ± 1.4 cm; corresponding values for Santiam River fish were 55.4 ± 10.4 g and 16.8 ± 1.2 cm. The wet weight of the survivors in the embryo–early juvenile test averaged 0.585 g based on batch weights of 1,147 fish.

The chemistry of well water used in the laboratory is given by Samuelson (1976). Water was sterilized with ultraviolet light, aerated to average dissolved oxygen concentration above 8.7 mg/liter, and adjusted to one of three constant temperatures and one of two total hardnesses. Median pHs were 6.9–7.1 among tests. Total alkalinity (as $CaCO_3$) averaged within 3 mg/liter of total hardness at low values (nominal 25 mg/liter) of hardness, and within 12 mg/liter at high values (nominal 125 mg/liter). Dissolved oxygen, pH, total alkalinity, and total hardness were measured daily from at least one aquarium containing fish by methods of APHA et al. (1971) and USEPA (1974), and at least weekly for each metal concentration. Water temperature was recorded continuously and averaged every 3 hours.

Analytical reagent grade zinc chloride was used; exposure concentrations are expressed as Zn^{++} ions. Stock solutions were acidified with 0.1 ml concentrated nitric acid per liter. Toxican concentrations were chosen on the basis of available toxicity data (Brown 1968; Benoit and Holcombe 1978; Chapman 1978a, 1978b; Chapman and Stevens 1978; Spehar et al. 1978). Zinc in water from one tank containing each concentration was measured daily by flame spectrophotometry; absorption was linear within the working range and analytical precision was ±3 μg/liter.

Embryo–Early Juvenile Test

The embryo–early juvenile test was conducted with Alsea River steelheads. Temperature was maintained at a nominal 12 C with chilling and heating units. Hardness was controlled at a nominal 25 mg/liter (as $CaCO_3$) by mixing normal well water with an appropriate amount of water that had been treated by reverse osmosis. Uniform water quality was necessary because variations affect zinc toxicity and negate comparisons among tests. Tests were conducted in 19-liter glass aquaria with a water volume of approximately 14 liters. A proportional diluter similar to that described by Mount and Brungs (1967) delivered six zinc concentrations (including control); the 2.15 liters per cycle at each concentration were split equally between duplicate aquaria. The 50% replacement time for aquaria was 20 minutes (Sprague 1969). Aquaria were surrounded by black plastic to control light and to lessen disturbance. Photoperiod was adjusted weekly to existing local day length with 30-minute dawn and dusk periods provided by rheostat-controlled incandescent lamps. Daytime light intensity (fluorescent) at the water surface was approximately 500 lux.

Equal numbers (about 140) of disinfected eggs were randomly assigned to an incubator in each of the 12 aquaria. Incubators were 10.2-cm diameter polyvinyl chloride pipe, 23 cm high and half filled with three layers of marbles (smallest at the top) to simulate streambed gravel. Water entered the bottom of the incubator, flowed up through the marbles and over the eggs, exited through perforations near the top, and drained out through the aquarium's screened standpipe drain.

Toxicant was introduced 1 day after the eggs and exposure continued for 72 days; nominal concentrations were approached in less than 2 hours. Unfertilized eggs were removed daily. At the eyed stage, eggs were shocked for about 7 seconds by vigorous turbulence from a hose; all viable eggs hatched within the next 3 days. At the swim-up stage, sac fry were liberated into the aquaria. At this time, and once weekly thereafter, six control fish were weighed less their yolk sacs. The weekly food ration (5.5% of body weight per day) was adjusted with sur-

[2] Mention of a product does not constitute endorsement by the Environmental Protection Agency.

vival. Loadings never exceeded 4.5 g fish/liter of aquarium water (0.05 g/liter-day) (Stephan 1975). Dead fry were removed during once-daily checks. Mortality after 72 days was the criterion for the MATC(EL). This MATC estimate was compared with published toxicity values for zinc to detect a possible acclimation effect (Sinley et al. 1974; Spehar 1976).

Ventilation Tests

Physical shielding of test chambers, photoperiod, temperature, and low nominal hardness for the ventilation tests were maintained as for the embryo–early juvenile test. High hardness was created in a steel tank filled with limestone; well water injected with CO_2 flowed through the tank and was subsequently aerated to neutralize pH.

Electrode chambers for ventilation tests were similar to those described by Spoor et al. (1971). One positive and one negative electrode in each chamber picked up muscle action potentials from buccal and opercular cavity movements, which were relayed to a four-channel strip chart recorder. Water and zinc solutions were delivered by a continuous-flow serial diluter similar to that described by Garton (1980). The flow rate into each electrode chamber was 200 ± 6 ml/minute. Chambers measured 38.1 × 15.2 cm and 15.2 cm long and had volumes of 6.64 liters, resulting in a 50% replacement time of less than 1 hour (Sprague 1969). All chambers were fitted with white plexiglass lids and painted white to reduce disturbance; light intensity at the water surface was about 165 lux.

Sixteen steelhead smolts were randomly assigned to tests, one to each chamber, four to each zinc concentration. Ambient temperature of the holding-tank water increased during the course of the study from 8.5 to 18.0 C. Chamber temperatures were always within 2 C of ambient when the fish were introduced. These temperatures were gradually adjusted to the test temperatures by ≤2.5 C per 24 hours, and fish had 3–8 days to acclimate.

After chambers reached test temperatures, baseline ventilation rates of the fish were monitored every 2 hours for 24 hours before zinc was introduced. Each "sampling period" was 4 minutes per fish preceded by a 15-second start-up period. The four-channel recorder allowed four fish to be sampled simultaneously; each of the four was at a different zinc concentration. The four groups were sampled in sequence until all 16 fish had been monitored; intervals between sampling periods varied. After 24 hours, zinc was introduced to the chambers and exposure continued for 96 hours. For the first 20 hours sampling was done every hour, from 20 to 50 hours, every 2 hours, from 50 to 96 hours, every 4 hours. Sampling periods were 4 minutes per fish. Ventilation frequency was counted manually on the strip-chart records. Originally, we intended to monitor coughing frequency, but we were unable to identify coughs consistently or objectively.

Statistics

Percent mortality data from the embryo–early juvenile test were subjected to arcsin transformation and analysis of variance (Steel and Torrie 1960) to arrive at an MATC(EL) range. Ventilation frequencies were transformed to $\log_{10}$ before comparisons were made. This conversion is recommended (Snedecor and Cochran 1967) with effects that are proportional rather than additive. Differences between mean logs of ventilation rates before and after zinc was added were subjected to analysis of variance. When treatment effects were significant, Williams' (1971) multiple comparison test determined which zinc concentrations were responsible. This analysis presumes that ventilation rates behave in a dose–response manner.

Results and Discussion

Embryo–Early Juvenile Test

The MATC concept, originally described by Mount and Stephan (1967), is the most useful method at present for determining the potential that organisms will survive exposure to water-borne chemicals. It is based on experiments covering an entire life-cycle. Macek and Sleight (1977) and McKim (1977) point out that embryo–early juvenile exposures yield nearly identical results in much less time. Our data may approximate an MATC for steelhead but we will call it MATC(EL) to designate that it relates specifically to the embryo and early juvenile life stages.

Mortality in the embryo–early juvenile exposure ranged from 2.4 to 92.6% in individual aquaria (Table 1). Only the highest tested zinc concentration (819 μg/liter) caused significantly

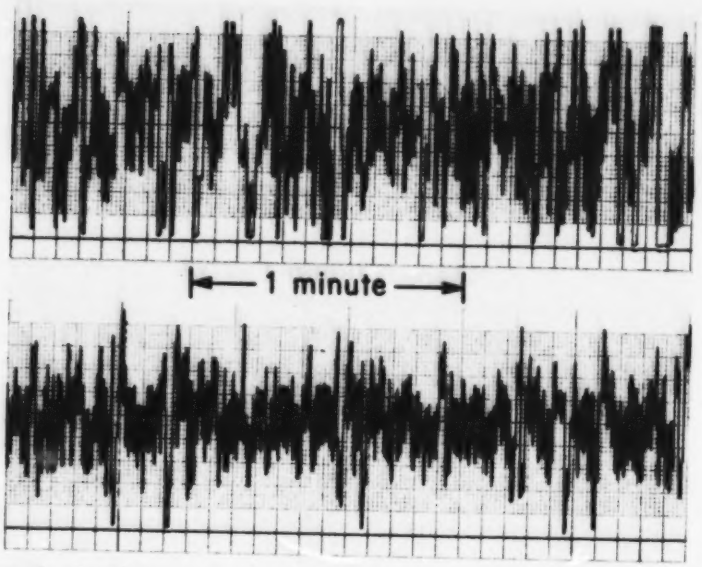

FIGURE 1.—*Examples of ventilation patterns of a steelhead before (top) and during (bottom) exposure to 176 μg Zn/liter in test 1. Note increase in breathing frequency caused by toxicant.*

greater mortalities than the control water, and an MATC(EL) of 444–819 μg Zn/liter is indicated. Final weights of survivors were not correlated with zinc concentrations in this test.

Our MATC(EL) agrees well with the MATC (534–1,360 μg Zn/liter) found by Holcombe et al. (1979) for brook trout *Salvelinus fontinalis*. Benoit and Holcombe (1978) found an MATC of 78–145 μg Zn/liter for fathead minnows *Pimephales promelas*; Spehar (1976) showed one of 26–51 μg/liter for flagfish *Jordanella floridae*; Sinley et al. (1974) reported an MATC of 140–260 μg/liter for rainbow trout *Salmo gairdneri*. All cited tests were done in soft water.

Sinley et al. (1974) and Spehar (1976) found that MATCs of fish first exposed as embryos were three or four times higher than those of fish exposed first as larvae. This acclimation may be caused by activation of enzymes during the resistant embryo stage that either eliminate zinc or control its tissue concentration (Giesy and Weiner 1977). It probably contributed to our MATC(EL). Under the same experimental conditions in this laboratory, Chapman (1978b) reported a 96-hour LC50 for steelhead parr and smolts of 136 and >651 μg/liter, respectively, rather less than our MATC(EL). In addition to demonstrating acclimation in our fish, this comparison indicates that an acute test is as sensitive for salmonids as a chronic test in soft water.

Ventilation Tests

The strength of the bioelectric signal picked up by the present system varied from 20 to 400 microvolts. This agrees well with the findings of Camougis (1960), Roberts (1964), and Spoor et al. (1971). Ventilation patterns of unstressed steelheads produced regular peaks and valleys on the strip-charts. The presence of zinc disrupted this pattern, sometimes affecting amplitude, sometimes frequency (Fig. 1). Although some investigators have reported ventilation amplitude in similar systems (van der Schalie 1977), amplitude cannot be measured in this sytem because it depends upon the distance of the fish from the electrodes. We evaluated only variations in rate, as did Maki (1979). Breathing rates were not correlated with fish size in these tests.

In the five tests conducted at 12 C and 25 mg/liter hardness (nominal), no group of fish significantly changed their ventilation rates at mean test concentrations of zinc below 144 μg/

TABLE 1.—*Zinc concentrations (mean ± SD) and percent mortality in embryo–early juvenile test with Alsea River steelheads. Concentrations with asterisks (*) embrace the estimated MATC(EL) (maximum acceptable toxicant concentration for embryos and early juveniles). Water temperature was 12.7 ± 0.4 C; total hardness was 27 ± 4 mg/liter as $CaCO_3$.*

Zinc concentrations (μg/liter)	Mean percent mortality (values of two replicates)
<5 (control)	9.6 (13.1, 6.2)
86 ± 6	3.4 (4.4, 2.4)
148 ± 11	4.9 (6.4, 3.4)
262 ± 20	8.0 (9.9, 6.2)
*444 ± 30	17.8 (20.0, 15.5)
*819 ± 50	85.6 (92.6, 78.7)[a]

[a] Significantly higher than control mortality by analysis of variance and Williams' multiple comparison tests.

liter; in one of these tests (3) a response concentration was not reached (Table 2). There was no discernable difference in the responses of Alsea and Santiam River fish. In combination, tests 1–5 indicate a ventilation response concentration (VRC, the lowest toxicant concentration that elicits an increased frequency) in the range of 117–144 μg/liter. This corresponds well with Chapman's (1978b) LC50 of 136 μg/liter for steelhead parr, and is well below our MATC(EL) of 444–819 μg/liter. Ventilation-rate tests seem at least as sensitive as chronic tests under these conditions.

Temperature may have affected the VRC, but the results are ambiguous. At 7 C (test 9), fish did not respond to 147 μg Zn/liter; at 17 C (test 10), they did not react to 141 μg/liter. However, both these concentrations, the highest tested at those temperatures, fall within the VRC deduced for 25 C, so a conclusion cannot be reached. There is disagreement in the literature about (or even if) temperature affects zinc toxicity (Cairns and Scheier 1957; Lloyd 1960; Brown 1968).

The VRC was affected by hardness; at 125 mg/liter hardness, ventilation rates were not affected by zinc concentration as high as 814 μg/liter (tests 6 and 7). In test 8, ventilation rates were elevated in fish exposed to 781 μg Zn/liter, however. That hardness decreases zinc toxicity has been pointed out elsewhere (Cairns and Scheier 1957; Lloyd 1960; Brown 1968; Holcombe and Andrew 1978). Although Lloyd (1960) stated that there is no constant ratio between the toxicities of zinc at two specific hardness values, it is known that calcium ions antagonize the action of zinc in fish (Skidmore 1964). We have not extended ventilation tests far enough yet to know if they adequately reflect toxicities in hard water.

Use of Ventilation Monitoring

Fish can acclimate to some toxicants during continuous exposure and their ventilation rates decline (Dandy 1967; Walden et al. 1970; Drummond et al. 1973; Maki 1979); this would negate the value of the ventilation test as an early warning indicator of toxicity in automatic biomonitoring systems. We found no evidence of acclimation in this study. When the 96-hour data were sub-divided into four 24-hour periods and analyzed statistically, the number of 49–72- and 73–96-hour periods containing significant increases in ventilation rate was as great as the number of 0–24- and 25–48-hour periods showing them. Acclimation might have occurred during a longer exposure period, and the possibility of acclimation should be examined for each monitoring system before it is used routinely.

This study supports other work showing that toxicant concentrations produced breathing aberrations at or near threshold chronic levels (Walden et al. 1970; Davis 1973; Drummond et al. 1973; Drummond et al. 1974; Maki 1979). Ventilation tests can detect effects at concentrations corresponding to chronic values derived from longer life-cycle or critical life-stage exposures. MacLeod and Smith (1966) found that increased coughing frequency (also, presumably, ventilatory frequency) indicates that the organism is undergoing a "loading stress" (Brett 1958) because the energy needed for normal maintenance is increased. Ventilation frequency reflects integrated stress on all organ systems. Without the behavioral interference created by electrode or cannulae implantations, this method gives an easily quantified measurement of the collective impact of all environmental factors; this cannot be achieved by continuous physical and chemical monitoring of individual variables.

In the present study, results obtained in 24 hours were similar to those obtained in 96 hours (acute test) or 72 days (embryo–early juvenile test). Therefore, estimates of safe chronic concentrations can be made very rapidly with this procedure. With proper precautions, ven-

TABLE 2.—*Test conditions for steelhead smolts exposed to zinc. Values are means ± SD; those marked with asterisks (*) embrace the VRC (ventilation response concentration) for each test.*

Test	Fish strain[a]	Temperature (C)	Total hardness (mg/liter)	Zinc concentration (μg/liter)	Mean ventilations per minute[b]	
					Before exposure	During exposure
1	A	12.0 ± 0.2	27 ± 3	11 ± 5	57	52
				51 ± 9	49	51
				*94 ± 16	54	52
				*176 ± 25[c]	55	62
2	A	12.1 ± 0.3	23 ± 1	2 ± 2	69	70
				34 ± 6	58	61
				*73 ± 5	64	63
				*144 ± 21[c]	57	72
3	A	11.8 ± 0.2	25 ± 5	3 ± 2	70	64
				38 ± 4	60	58
				76 ± 11	64	66
				117 ± 5	66	63
4	S	12.1 ± 0.1	22 ± 1	<2	56	51
				52 ± 3	61	60
				*115 ± 10	55	54
				*200 ± 8[c]	56	58
5	S	12.0 ± 0.1	22 ± 1	<5	53	47
				42 ± 9	50	46
				*91 ± 10	56	55
				*179 ± 22[c]	53	56
6	A	12.5 ± 0.9	124 ± 5	<10	62	64
				224 ± 29	60	55
				441 ± 44	63	68
				814 ± 86	65	64
7	A	12.3 ± 0.7	108 ± 28	<5	67	64
				224 ± 9	73	69
				440 ± 15	73	72
				814 ± 24	80	74
8	A	12.6 ± 0.9	119 ± 4	<5	79	75
				212 ± 27	71	63
				*419 ± 37	81	78
				*781 ± 66[c]	76	79
9	A	8.0 ± 0.3	26 ± 1	<5	53	52
				41 ± 5	49	50
				78 ± 4	58	55
				147 ± 8	53	54
10	A	17.2 ± 0.2	26 ± 1	<5	60	54
				33 ± 8	65	64
				72 ± 10	61	56
				141 ± 16	60	59

[a] A = Alsea River strain; S = Santiam River strain.
[b] Each number is a mean of four fish per treatment.
[c] This concentration produced a significantly elevated ventilation frequency when compared to that of the control fish by analysis of variance and Williams' multiple comparison tests.

tilation tests may be useful in developing water quality criteria.

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Robert Drummond helped with the early phase of the research. Personnel of the Toxicology Branch of the Environmental Protection Agency's Research Laboratory in Corvallis were generous with their time in providing advice and editorial review, especially Steven Hansen. The Laboratory's facilities staff built the multiplex switching device for use with the strip-

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